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(54) Title: IDENTIFICATION OF NOVEL DRUGS AND REAGENTS

(57) Abstract

Methods for identifying oligonucleotides capable of hybridizing with nucleic acid of an infectious agent are disclosed. In accordance with preferred embodiments, oligonucleotides capable of antisense interaction with RNA which is significant to a bodily state of an animal or which is contributory to a diseased state is identified. Therapeutic, diagnostic and research methods and compositions employing such oligonucleotides are provided. Prior knowledge of the sequence of nucleic acid in the infectious agent is generally not required.

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IDENTIFICATION OF NOVEL DRUGS AND REAGENTS

FIELD OF THE INVENTION

This invention relates to the identification, preparation and use of novel therapeutic, diagnostic and research compositions and to methods for their use. More particularly, this invention relates to novel antisense compositions, compositions which can interact with nucleic acids by hybridization to effect their function and to cause changes in biological activity thereby. Methods for identifying such antisense compositions are provided which lead to novel therapeutic, diagnostic and research compositions and to methods for diagnosis, treatment and experimentation.

BACKGROUND OF THE INVENTION

It is well known that most of the bodily states in mammals including most disease states, are effected by proteins. Such proteins, either acting directly or through their enzymatic functions, contribute in major proportion to many diseases in animals and man. Classical therapeutics has generally focused upon interactions with such proteins in efforts to moderate their disease causing or disease

potentiating functions. Recently, however, attempts have been made to moderate the actual production of such proteins by interactions with molecules that direct their synthesis, intracellular RNA. By interfering with the production of proteins, it has been hoped to effect therapeutic results with maximum effect and minimal side effects. It is the general object of such therapeutic approaches to interfere with or otherwise modulate gene expression leading to undesired protein formation.

One method for inhibiting specific gene expression which has been adopted to some degree is the "antisense" approach, where oligonucleotide analogues complimentary to a specific, target, messenger RNA. A number of workers have reported such attempts. Pertinent reviews include C.A. Stein & J.S. Cohen, *Cancer Research*, vol. 48, pp. 2659-2668 (1988); J. Walder, *Genes & Development*, vol. 2, pp. 502-504 (1988); C.J. Marcus-Sekura, *Anal. Biochemistry*, vol. 172, 289-295 (1988); G. Zon, *Journal of Protein Chemistry*, vol. 6, pp-131-145 (1987); G. Zon, *Pharmaceutical Research*, vol. 5, pp. 539-549 (1988); A. R. Van der Krol, J.N. Mol, & A.R. Stuitje, *BioTechniques*, vol. 6, pp. 958-973 (1988) and D.S. Loose-Mitchell, *TIPS*, vol. 9, pp. 45-47 (1988). Each of the foregoing provide background concerning general antisense theory and prior techniques.

Prior attempts at antisense therapy have provided oligonucleotides or oligonucleotide analogues which are designed to bind in a specific fashion to--which are specifically hybridizable with-- a specific mRNA by

hybridization. Such analogues are intended to inhibit the activity of the selected mRNA-- to interfere with translation reactions by which proteins coded by the mRNA are produced-- by any of a number of mechanisms. The inhibition of the formation of the specific proteins which are coded for by the mRNA sequences interfered with have been hoped to lead to therapeutic benefits.

A number of chemical modifications have been introduced into antisense oligonucleotides to increase their therapeutic activity. Such modifications are designed to increase cell penetration of the antisense oligonucleotides, to stabilize them from nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotide analogues in the body, and to improve their pharmacokinetic properties. At present, however, no generalized antisense oligonucleotide therapeutic or diagnostic scheme has been found. Prior efforts have been limited by the inability to deliver a sufficient quantity of antisense oligonucleotide into appropriate cells for effective activity to take place. The activity of the antisense oligonucleotides presently available has not been sufficient for effective therapeutic, research reagent, or diagnostic use in any practical sense. Accordingly, there has been and continues to be a long-felt need for oligonucleotides and oligonucleotide analogues which are capable of effective therapeutic and diagnostic antisense use.

This long-felt need has not been satisfied by prior work in the field of antisense oligonucleotide therapy and

diagnostics. Others have failed to provide materials which are, at once, therapeutically or diagnostically effective at reasonable rates of application. In particular, there has been no generalized methodology for identifying oligonucleotides which are capable of effectively treating infectious agents. All known, prior methodologies for identifying such nucleotides have required prior knowledge of the nucleic acid sequence for that portion of nucleic for which hybridization is desired. It is therefore greatly to be desired to identify effective antisense oligonucleotides for infectious agents, which identification does not require such prior knowledge of the nucleic acid sequence.

OBJECTS OF THE INVENTION

It is an object of this invention to provide methods for identifying oligonucleotides useful in antisense therapy of diseased states.

A further object is to identify oligonucleotides useful for the determination of the status of bodily functions of animals.

Yet another object is to identify oligonucleotides which are useful as research reagents such as for blocking gene expression of particular RNA molecules.

An additional object of the invention is to identify loci of nucleic acids which control the expression of polypeptides having a significant effect on a bodily function of an animal.

A further object is to identify and provide such

oligonucleotides without the need for prior knowledge of the sequence of nucleic acid for which hybridization is desired.

Other objects will become apparent to persons of ordinary skill in the art from a review of the present specification.

SUMMARY OF THE INVENTION

Although antisense oligonucleotides are currently being investigated as potential therapeutic agents, it is not clear how to select the best possible nucleotide sequence to inhibit the expression of a particular mRNA. Moreover, in many cases it is even difficult to decide what gene to target for antisense inhibition to alter the course of a disease. The method of the present invention overcomes these difficulties.

In accordance with this invention, methods are provided for identifying oligonucleotides capable of hybridizing with nucleic acid of an infectious agent comprising providing a plurality of vectors which include substantially randomly sequenced oligonucleotides and incorporating those vectors into cells. The cells infected with the infectious agent and provided with conditions for growth of the cells. Cells which are resistant to the infection are then identified. Preferably, the nucleic acid sequence of the included oligonucleotide is determined for those cells resistant to the infection.

In accordance with preferred embodiments, the substantially randomly sequenced oligonucleotides are prepared

through solid state synthesis or otherwise and comprise from about 10 to about 100 nucleic acid subunits. Alternatively, cDNA or genomic DNA preferrably from an infectious organism may be used in the preparation of the vectors. Such DNA is preferrably fragmented to provide a plurality of fragmentary portions of the DNA having numbers of subunits in the range of from about 50 to a few thousand with from about 100 to about 1000 being convenient and preferred. The cells used are preferably selected to be easily infectable by the infectious agent.

In accordance with other embodiments, reagents are prepared having as at least a component thereof the nucleic acid sequence of the inserted oligonucleotide which has been identified as coming from a cell resistant to the infectious agent. Therapeutic, diagnostic and research compositions and kits are prepared from such reagents and methods of therapeutics, diagnosis and research employing them are comprehended as being within this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a schematic structure of the vector ISIS RG-1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of identifying anti-sense oligonucleotide sequences that inhibit gene expression of an infectious or other agent. Expression vectors containing oligonucleotide sequences formed by random

synthesis, derived from an infectious agent by treatment of the DNA or cDNA of the agent with DNAase, or by comminution, or derived from the mRNA of cells infected with the infectious agent are transformed into cells. The cells are then infected with the infectious or other agent. Oligonucleotides that inhibit gene expression of the infectious agent and which are thus useful in suppressing the effects of the agent are then selected by selecting cells that do not exhibit effects of infection with the infectious agent; for example, the cells do not die, the cells grow at an increased rate, or exhibit other behavior suggestive of modulation of the infection. Once such cells have been identified, the sequence of the oligonucleotide having the ameliorative effect is identified. Identification may be accomplished by reconvering the vector and sequencing the region containing the inserted nucleic acid material.

The cells, expression vectors and method of selecting for the oligonucleotide sequence will be determined by the type of infectious or other agent. For example, if it is desired to identify anti-sense oligonucleotides to a herpesvirus, the cell type chosen will be one that can be infected with the particular herpesvirus, and the expression vector will be one that is compatible with the cell chosen.

In accordance with this invention, the term "random" as applied to sequences of nucleic acid has several related meanings. Thus, truly random oligonucleotides formed through solid state synthesis are comprehended. Even this protocol need not be completely and statistically random in fact,

however. Thus, enrichment of oligonucleotides in certain bases may be desired in accordance with some embodiments of this invention.

A further meaning of the term "random" in the present context relates to the use of genomic DNA from organisms, especially an infectious agent. Such genomic DNA is harvested from the organism in a conventional way and then either comminuted or subjected to DNAase attack to form a plurality of fragments of the DNA. While not truly random in that the parent DNA had a definate sequence, the sequence is generally unknown and its fragmentation is generally uncontrolled.

A further meaning of the term "random" as applied to this invention relates to complementary DNA or cDNA. Complementary DNAs can be prepared for all or some RNAs of an organism, especially an infectious organism. The cDNA may then be inserted into a vector and used otherwise as discussed herein.

It will be understood that the use of random oligonucleotides as intended by this invention encompasses all of the foregoing alternatives. The oligonucleotide fragments of portions are generally of a size as to be effective in the performance of this invention. Generally, from about 20 to 100 bases are employed for the synthetically generated random sequences and from about 100 to a few thousand base units are employed with genomic DNA or cDNA.

The manipulation of expression vectors and oligonucleotides and the transformation of cells is performed

according to standard techniques such as may be found in Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York, 1982.

Expression vectors suitable for use in the methods of the invention include commercially available plasmid vectors such as pMAMM-NEO (Clonetech) and other expression vectors. It is preferable that the plasmid expression vector contain at least one gene to allow construciton and recovery of the vector, such as an antibiotic resistance gene such as the ampicillin resistance gene. It is also preferable that the plasmid expression vector contain an inducible promoter and transcriptional initiations region to express the random or semi-random sequences and a polyadenylation signal to stabilize the random sequences. This may or may not include an intron for more efficient expression.

A preferred plasmid expression vector is ISIS RG-1 which is illustrated schematically in figure 1. This vector contains the neomycin gene for G418 selection of stably transformed cells in culture and the ampicillin gene for bacterial amplification. Inserts can be directionally cloned by cutting at the Hind III and Xba I sites, releasing a stuffer fragment. The Hind III site is immediately 3' to the RSV promoter and the Xba I site is immediately 5' of the bovine growth hormone (BGH) poly (A) site.

Oligonucleotides for use in the methods of the invention may be obtained from several sources. Random sequences can be prepared by use of a DNA synthesizer to generate a length of DNA with an equimolar mixture of A, G,

C, T at each position. The complimentary strand can be generated using a DNA primer and DNA polymerase. By making DNA ends of the random sequence complementary to the overhangs left by restriction enzyme cleavage it is possible to clone the random mixture into the expression vector. For semi-random oligonucleotides, the DNA of the infectious agent may cut into short pieces by sheared or treatment with DNAase. For example, Herpes DNA may be sheared or DNAase treated to small sizes and "shotgun cloned" into the expression vector. Some of the sequences expressed would be antisense to Herpes genes. Alternatively, a cDNA library from mRNA isolated from infected cells is created. The cDNA is then cloned directionally cloned into the expression vector such that RNAs are produced in an antisense orientation. This approach would identify new genes that are key to successful infection.

Once expression vectors containing oligonucleotides are constructed, the expression vectors are inserted into cells susceptible of infection with the infectious agent. The cells may already be infected with the infectious agent or they may be infected with the infectious agent after insertion of the expression vector. The expression vectors are inserted into the cells by standard methods, such as calcium phosphate transfection or electroporation.

The cells are then cultured and oligonucleotides that are anti-sense to mRNA of the infectious agent are selected. Selection methods will depend on the type of cell and the effects of the infectious agent. For example, neommyosin resistant cells can be propagated to confluency in

10 cm tissue culture dishes. When confluency is reached the entire population is infected with HSV-1 at a low MOI. Media is changed at frequent intervals following the infection to reduce the frequency of secondary infection. Cells which survive the infection are grown to confluency, then infected a second time in the same manner. This process is repeated until all cells survive an infection or until individual resistant colonies are selected.

Once cells containing the desired oligonucleotides have been selected, the sequences of the oligonucleotides having activity are determined. Identification of the oligonucleotide sequence may be accomplished by several methods. The oligonucleotide may be removed from the expression vector and sequenced. Alternatively, the oligonucleotide sequence may be amplified in the cell by polymerase chain reaction, using appropriate primers, and the sequence of the amplified sequence determined.

Examples

Example 1 Vector Construction

The following method describes a scheme to generate random and semi-random antisense messages to screen for sequences to specifically inhibit HSV infection of mammalian cells in culture. This may be done by three different means. 1) random-synthetic oligomers of defined length, 2) semi-random- produced by shearing genomic DNA, and 3) by generating a cDNA library from HSV infected cells. In each case the vector used will be ISIS RG-1. This vector contains the

neomycin gene for G418 selection of stably transformed cells in culture and the ampicillin gene for bacterial amplification. Inserts can be directionally cloned by cutting at the Hind III and Xba I sites, releasing a stuffer fragment. The Hind III site is immediately 3' to the RSV promoter and the Xba I site is immediately 5' of the bovine growth hormone (BGH) poly (A) site. (see fig. 1)

I. RANDOM SEQUENCES A random population of oligonucleotides will be produced such that the 5' end of each oligomer consists of the Hind III site, d(CAAGCTTG). This is followed by approximately 25 nt of random sequence, then the tail sequence d(TCTAGAGAAAAA), creating an Xba I site and poly A tail. A primer complementary to the 3' end sequence, 5'd(TTTTTCTCTAGA)3', will then be used as a primer for the synthesis of complementary strands to each of the random oligomers produced creating double strand molecules. The population of oligomers will then be subject digestion with Hind III and Xba I to give cohesive ends compatible with the vector.

II. RANDOM GENOMIC FRAGMENTS The prepared vector with Hind III and XbaI ends will be Klenow filled , creating blunt ends. This will be followed by treatment with ligase to recircularize the molecule. This restores the Xba I site while eliminating the Hind III site. The vector is then cut with Xba I and the ends phosphatased to prevent recircularization. Insert will then be generated by cutting total genomic DNA with varying concentrations of DNAse I, the isolating the products of desired size on a preparative

agarose gel. Ends of the sheared fragments will then be repaired with T7 DNA polymerase and Xba I linkers, d(GCTCTAGAGC), blunt end ligated, followed by cutting the linked inserts with Xba I and cloning of the inserts directly into the vector.

III. cDNA FRAGMENTS mRNA will be isolated from cells infected with HSV. To directionally clone these sequences into the RG-1 vector, the poly A+ mRNA will be primed with a Hind III primer/adaptor d(CCAAGCTTGG$(T)_{15}$). Following annealing of the primer, cDNA will be produced essentially as outlined by Gubler and Hoffmann (fig. 2).

Transformation/Selection Once the efficiency of the constructions has been determined, the plasmids will be transfected as a population into CV-1 or HeLa cells using the method of Chen and Okayama. Stable transformants will be selected on the basis of resistance to G418. The population of stably transformed cells will then be infected with HSV. Surviving colonies presumably will be expressing mRNA which is interacting to specifically block HSV infection. The plasmid DNA responsible for the effect can then be isolated from the transformed cells by PCR using Hind III and Xba I primers.

WHAT IS CLAIMED IS:

1. A method for identifying an oligonucleotide capable of hybridizing with nucleic acid of an infectious agent comprising:

(a) providing a plurality of vectors which include substantially randomly sequenced oligonucleotides;

(b) incorporating the vectors into cells;

(c) infecting the cells with the infectious agent; and

(d) providing conditions for growth of the cells.

2. The method of claim 1 further comprising:

(e) identifying cells which are resistant to the infection; and

(f) determining the nucleic acid sequence of the included oligonucleotide in a resistant cell.

3. The method of claim 1 wherein the oligonucleotides included in the vectors comprise from about 10 to about 100 nucleic acid subunits and are prepared through a solid state synthetic technique.

4. The method of claim 1 wherein the oligonucleotides included in the vectors derive from genomic DNA.

5. The method of claim 4 wherein the oligonucleotides included in the vectors comprise from about 100 to about

10,000 base units.

6. The method of claim 1 wherein the oligonucleotides included in the vectors derive from cDNA.

7. The method of claim 6 wherein the oligonucleotides included in the vectors comprise from about 100 to about 10,000 base units.

8. The method of claim 1 wherein the cells are selected to be infectable by the infectious agent.

9. The method of claim 1 wherein the infectious agent is a herpesvirus and the cells are epithelial cells.

10. A composition comprising oligonucleotide substantially including the nucleic acid sequence of the inserted oligonucleotide in a resistant cell in accordance with claim 1.

11. The composition of claim 10 in a pharmacologically acceptable carrier.

12. A kit for determining the condition of a bodily state of an animal comprising the composition of claim 10.

13. An oligonucleotide identified in accordance with the method of claim 1.

14. An oligonucleotide having a sequence determined in accordance with claim 2.

15. A pharmaceutical composition comprising an oligonucleotide in accordance with claim 13.

16. A pharmaceutical composition comprising an oligonucleotide in accordance with claim 14.

17. A method for treating a disease caused by an infectious agent comprising administering to an animal suspected of having the disease an oligonucleotide identified in accordance with claim 1.

18. A method for treating a disease caused by an infectious agent comprising administering to an animal suspected of having the disease an oligonucleotide having a sequence determined in accordance with claim 2.

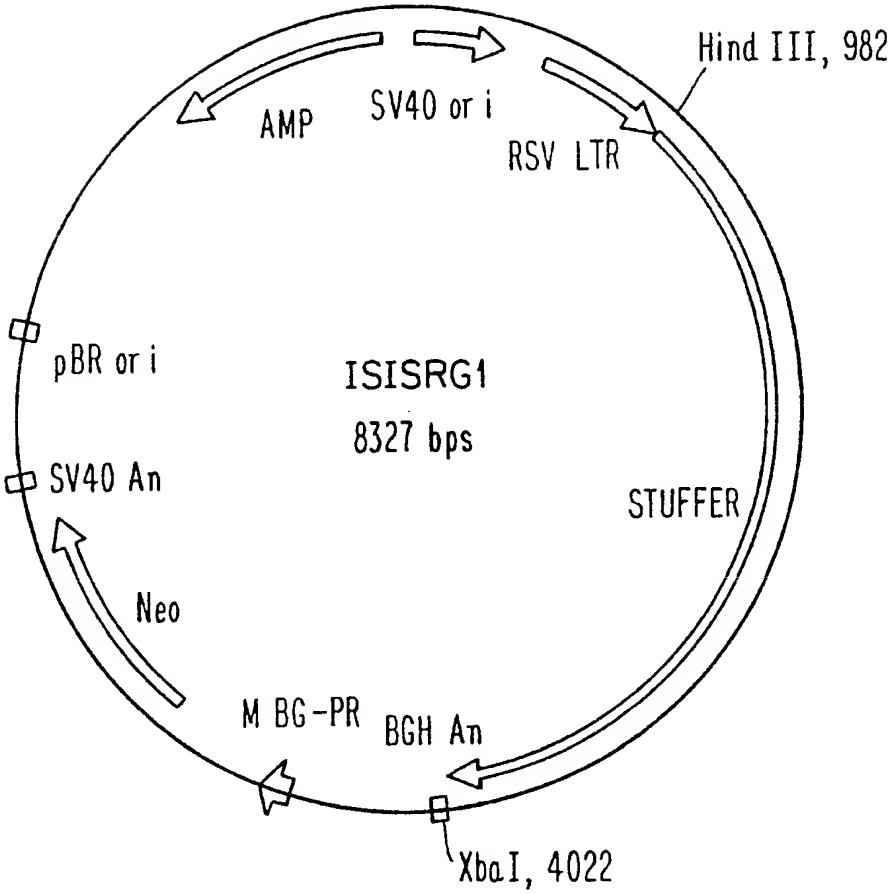
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Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/02574

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68
U.S. Cl.: 435/6

II FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S. CL.	435/6, 172.3, 320.1; 436/501, 811; 514/44; 935/77, 78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

APS and Dialog data bases

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
Y	Pharmaceutical Research, Vol. 5, No. 9, issued 1988, ZON, "Oligonucleotide Analogues as Potential Chemotherapeutic Agents", pages 539-549, see the section "Biological Effects of Oligonucleotides and Analogues there of.	1-16
Y	G.M. Hasler et al., "A Rapid Quantitative Bioassay Based on the Human Immuno-deficiency Virus Trans-Activator" published 1989 by Mary Ann Liebert, Inc., see pages 507-516.	1-16
Y	Cancer Research, Vol. 48, issued 15 May 1988, STEIN ET AL., "Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review", pages 2659-2668, see entire document.	1-16
Y	Analytical Biochemistry, Vol. 172, issued 1988, Marcus-Kekura, "Techniques for Using Antisense Oligodeoxyribonucleotides to Study Gene Expression", pages 289-295, see abstract, Types of Antisense Oligomers and Use of Antisense Oligomers in Cell Culture.	1-16

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 June 1991	04 SEP 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Amelia B. Yarbrough

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. The method of identifying oligonucleotides,composition of nucleotides and oligonucleotides classified in class 435, subclass 6 and class 536, subclass 27. (Claims 1-16)

II. The method of treating classified in class 514 subclass 44.(Claims 17,18)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: Telephone practice

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.